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SYNTHESIS OF N-DESMETHYL PNQX: ELABORATION INTO AN IMMUNOGENIC CONJUGATE AND A RADIOIODINATED LIGAND; RADIOIMMUNOASSAY FOR THE AMPA/NMDA ANTAGONIST, PNQX

Christopher F. Bigge,* C. Stephen Yi, and Gerald D. Nordblom

Departments of Chemistry, and Pharmacokinetics and Drug Metabolism

Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co.

2800 Plymouth Road, Ann Arbor, MI 48105

Abstract: A nine-step synthesis of N-desmethyl PNQX **5** and its subsequent elaboration into analogs, a porcine thyroglobulin immunogenic conjugate **8**, and a radioiodinated analyte **10** are described. These compounds were used for the development of a specific radioimmunoassay for PNQX, a potent neuroprotective agent with mixed AMPA/NMDA glycine site antagonist activity. © 1997 Elsevier Science Ltd.

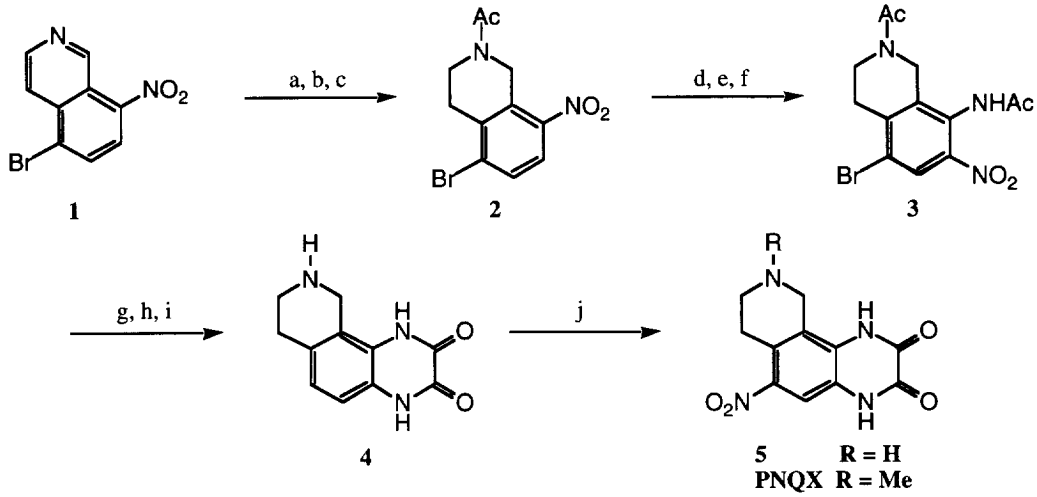
PNQX is a potent antagonist of both the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartic acid) excitatory amino acid receptors (IC_{50} = 63 nM at AMPA and 370 nM at the NMDA glycine site).[1] In the original literature, PNQX (1,4,7,8,9,10-hexahydro-9-methyl-6-nitropyrido[3,4-*f*]-quinoxaline-2,3-dione) showed functional antagonism of both AMPA and NMDA receptors in neuronal cultures and in the rat cortical wedge. It was postulated that the increased potency demonstrated by PNQX compared to NBQX (2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline)[2], a selective AMPA receptor antagonist (IC_{50} = 52 nM at AMPA), in the maximal electroshock anticonvulsant assay (ED_{50} = 0.44 mg/kg versus 13.1 mg/kg, respectively) was due to its combined action at AMPA and NMDA receptors.[1] More recently it has been reported that preadministration with probenecid can enhance the anticonvulsant effect of PNQX approximately ten-fold and extend its duration of action dramatically.[3] Evidence from in vivo experiments suggested that the effects of probenecid on the overall efficacy of PNQX are due to an action at a brain transport site(s) that results in increased concentrations in the brain relative to concentrations in the plasma. In a reperfusion model of ischemic stroke in which the middle cerebral artery is blocked for three hours, and then unclipped to allow reoxygenation, PNQX was shown to significantly reduce cortical infarct when given as an intravenous infusion.[4] These and other data have contributed to a continued interest in the development of PNQX as a clinical candidate for disorders benefitting from neuroprotective activity.

For clinical candidates it is essential to establish pharmacokinetic parameters. The quinoxalinedione moiety of PNQX has a chromaphore that can be easily observed by UV detection. An HPLC assay was developed that allows PNQX concentrations to be determined in plasma. However, the sensitivity of the HPLC assay is not sufficient to detect PNQX in cerebral spinal fluid or from brain microdialysis techniques. Because we were interested in correlating brain interstitial concentrations of PNQX to its pharmacological action as an excitatory

amino acid antagonist, analogs of PNQX were designed to enable us to develop a specific radioimmunoassay for PNQX.

Three primary interaction sites were identified for the binding of quinoxalinediones in an AMPA pharmacophore model: (i) a coulombic interaction between the tautomeric 2-oxo moiety of the quinoxalinedione and the receptor; (ii) a hydrogen bond donor from N-4 of the quinoxalinedione and the receptor; and (iii) a specific interaction between the 6-nitro group and the receptor.[1] Due to the importance of these interactions, it was essential to retain these primary functional groups when designing analogs for a radioimmunoassay (RIA). That left N-9 as the obvious choice for attachment of a tether in which to link a protein conjugate and to attach a radiolabeled analyte. Herein are described preparation of the key compounds that were utilized to develop the PNQX RIA including the porcine thyroglobulin-PNQX conjugate **8** used to immunize rabbits to generate specific antibodies, and an iodinated tyramine analog **10** that competes with PNQX for the specific antibody binding site.

Scheme 1.



Reagents: a. CH_3SO_3H, $CHCl_3$ b. $NaCNBH_3$, HCOOH c. Ac_2O d. H_2, RaNi e. Ac_2O f. $HONO_2$, TFA g. 3 N HCl, Δ h. H_2, 20% Pd-C i. $(COOH)_2$, 3 N HCl, Δ j. $HONO_2$, TFA

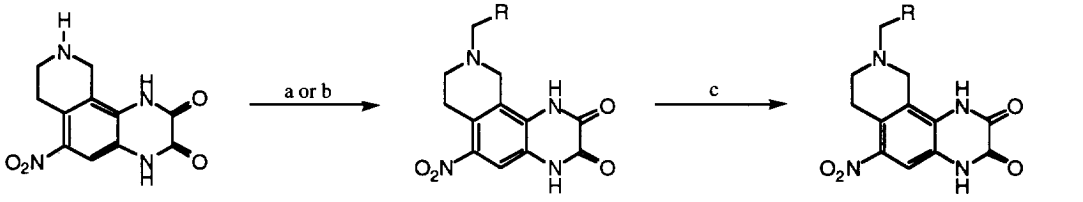
Chemistry

N-Desmethyl PNQX (**5**) is the key intermediate required for the preparation of a range of N-9 substituted analogs. Preparation of **5** involved a nine-step linear synthesis that began with the conversion of 5-bromo-8-nitroisoquinoline[1] **1** to its methanesulfonic acid salt, reduction with sodium cyanoborohydride in formic acid solution to the tetrahydroisoquinoline, and protection of the resulting amine to give the acetamide **2**. The 8-nitro moiety of **2** was selectively hydrogenated using Raney nickel as catalyst and treated with acetic anydride to give a

bisacetamide. Nitration gave selectively the 7-nitro adduct **3** as expected. Both N-acetyl groups were removed by acidic hydrolysis, and hydrogenation with 20% palladium on carbon reduced the 7-nitro group and removed the 5-bromo in one step. Heating with oxalic acid in 3 N HCl provided the parent piperidinyl-fused quinoxalinedione **4**. Consistent with previous findings in this ring system[1], mild nitration conditions gave exclusively the 6-nitro adduct **5**.

It was initially envisioned that the conjugate for eliciting the antibody could embody either a short or a longer chain tether. The synthesis of these is shown in Scheme 2. In each case alkylation proceeded in good yield in DMF (60 °C) with a trialkylamine base. In the first instance, reaction of methyl bromoacetate (diisopropylethylamine) with **5** gave the N-9 acetate **6a** in 76% isolated yield. For the preparation of the longer chain adduct **6b**, **5** was treated with 4-(2-bromo-acetylamino)-butyric acid methyl ester and triethylamine. The methyl esters were hydrolyzed in THF with either 1 N NaOH, or potassium trimethylsilanolate and acidified to give the free acids **7a** and **7b**.[5]

Scheme 2.



5 **6a R = $COOCH_3$** **7a R = COOH**

6b R = $CONH(CH_2)_3COOMe$ **7b R = $CONH(CH_2)_3COOH$**

Reagents: a. $BrCH_2COOMe$, $(iPr)_2NEt$ b. $BrCH_2CONH(CH_2)_3COOMe$, NEt_3 c. 1 N NaOH or $KOSiMe_3$, then 1 N HCl.

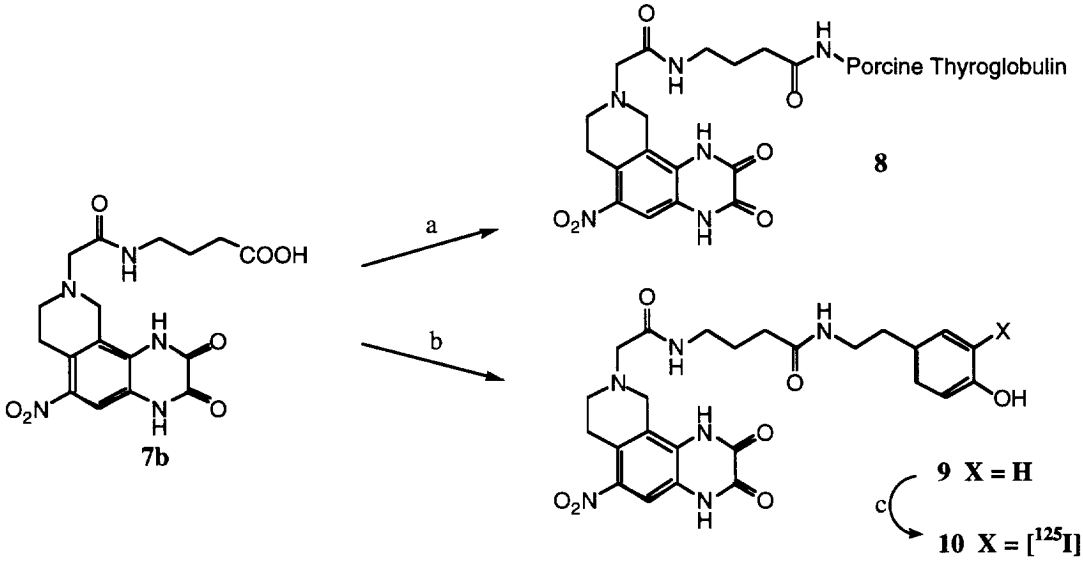
A porcine thyroglobulin conjugate **8** was prepared using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) as the coupling reagent to activate **7b**. Upon completion, the reaction mixture was dialyzed for 5 days with daily changes of 0.9% NaCl solution. The tyramine adduct **9** was prepared by treating a solution of **7b** in DMF with EDAC, triethylamine, and hydroxybenztriazole (HOBT) to form the activated ester, followed by the addition of tyramine. Radioiodination proceeded as expected using aqueous $Na[^{125}I]$ and chloramine-T as an initiator. The radioiodinated adduct **10** was purified by reverse-phase HPLC. Analytical and spectral data for products are noted.[6]

Radioimmunoassay (RIA)

Details of the preparation and harvesting of the PNQX antibody, as well as the development and validation of the resulting radioimmunoassay will be described in detail in a full paper. The PNQX RIA has been used successfully to measure plasma concentrations of PNQX, and to measure brain interstitial concentrations of

PNQX obtained by brain microdialysis following systemic administration of the drug. Briefly, the porcine thyroglobulin conjugate (200 μg in 1 mL emulsion containing 400 μg of the mitogenic agent, Adjuvax) was administered to six female New Zealand White rabbits in three successive injections with 14 day intervals. Serum was obtained from the rabbits seven to ten days following the last immunization. Serum from rabbit 305 gave the highest titer and was used to develop and validate the PNQX radioimmunoassay. A dilution of 1:14,000 of antiserum was required to bind 50% of added radioiodinated label.

Scheme 3.



Reagents: a. Porcine Thyroglobulin, EDAC, H_2O b. EDAC, HOBT, NEt_3, tyramine c. Na[^{125}I], chloramine-T

The PNQX RIA is based upon the ability of unlabeled PNQX to compete with the [^{125}I]-labeled tyramine adduct **10** for the antibody. Standard curves were constructed by plotting the logit-transformed percentage of radiolabel bound versus the log of the PNQX concentration. Samples, either appropriate standards or unknowns, were incubated with known amounts of antibody and radiolabelled **10**. After 2.5 h at 23 °C, the antibody-bound fraction was precipitated with sheep antirabbit gamma globulin antibody. The supernatant was decanted and the precipitate was counted in the gamma counter. The within-assay precision and accuracy of the RIA procedure was determined using six quality control samples run in duplicate at three concentrations in each assay run. Replicate analysis of the quality controls had a relative standard deviation of 3.5 - 13.1% and a percent relative error of -8.5 to 3.8%.

Using a 1:14,000 dilution of antiserum from rabbit 305 and approximately 50,000 cpm of radiolabel/tube, the ED_{80} or the mass of standard need to displace 20% of the label was 7.5 picogram per tube. The limit of quantitation

of the resulting standard curve defined as the lowest concentration standard, was determined to be 1.95 pg/tube of PNQX. Using 20 µL of sample matrix, this is equivalent to a sensitivity of 97.7 pg/mL of PNQX. The assumption is that the required neuroprotective concentration of PNQX in the interstitial fluids of the CNS is approximately equal to that of its IC_{50} at AMPA receptors. With a molecular weight of 279 and an IC_{50} = 63 nM at AMPA receptors, then this predicts that a brain concentration of 17.6 ng/mL is required for efficacy. These data suggest that the assay provides ca. 200-fold greater sensitivity than that required to detect expected neuroprotective concentrations, not withstanding the limitations of probe recovery using brain microdialysis techniques.

Discussion

The PNQX RIA was designed specifically for the detection of PNQX in biological fluids and tissues. A preliminary investigation to determine the absolute selectivity of this assay with the desmethyl PNQX **5** showed 91.5% crossreactivity. This activity is not surprising since **5** contains all of the key structural features of PNQX including the three primary interaction sites required for AMPA/glycine site binding. A more comprehensive examination of cross reactivity has been completed, and will be published as part of the complete description of the development and validation of the RIA. In this study, methods for the preparation of key compounds used for the development of an RIA for PNQX are described. The RIA has been useful for rapidly determining plasma levels in animals in which PNQX has been administered, thus providing pharmacokinetic and pharmacodynamic information. Crossreactivity with compounds containing the 6-nitroquinoxalinedione template has been noted, but there are differences between the antibody binding site and the AMPA and/or NMDA glycine sites. These differences were anticipated since the antibody site was obtained from **8** which contains a 10 atom tether to the immunogenic protein. Other aspects and applications of the PNQX RIA will be revealed in more detail in the full paper.

References and Notes

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5. **7a** was originally designed as a potential short tether derivative to make the protein conjugate and be converted into a radioanalyte, but proved unnecessary since **7b** worked well. **7a** is a very potent AMPA receptor antagonist (IC_{50} = 80 nM) that is essentially equipotent to PNQX. **7b** was ten-fold weaker at the AMPA receptor (IC_{50} = 890 nM), which probably reflects steric limitations in the region of the N-9 tether.

6. ^{1}H NMR and mass spectral data were consistent with assigned structure. Analytical data were within ±0.4 for C, H, N, except as noted. Compound **9** contained some starting material **7b**; **9** was radioiodinated and then purified by HPLC to give pure **10**. IUPAC nomenclature is used below.

- 1-(5-Bromo-8-nitro-3,4-dihydro-1H-isoquinolin-2-yl)-ethanone (**2**), mp 159 - 161 °C.
- N-(2-Acetyl-5-bromo-7-nitro-1,2,3,4-tetrahydro-isoquinolin-8-yl)-acetamide (**3**), mp 173 - 175 °C.
- 5-Ethylaminomethyl-6-methyl-1,4-dihydro-quinoxaline-2,3-dione (**4**), mp 294 - 297 °C.
- 5-Ethylaminomethyl-6-methyl-7-nitro-1,4-dihydro-quinoxaline-2,3-dione (**5**), Yellow solid, mp 269 - 272 °C (dec); ^{1}H NMR (400 MHz, TFA) δ 3.694 (2H, br s), 3.832 (2H, br s), 4.891 (2H, br s), 8.334 (1H, s); MS (CI) 263 (M^+); Anal. calcd for $C_{11}H_{10}N_4O_4$:1.25 H_2O: C, H, N.
- [Ethyl-(6-methyl-7-nitro-2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-ylmethyl)-amino]-acetic acid methyl ester (**6a**). Orange solid, mp 260-263 °C (dec); ^{1}H NMR (400 MHz, DMSO) δ 2.74 (2H, t), 2.98 (2H,t), 3.44 (2H,s), 3.62 (3H, s), 3.70 (2H, s), 7.69 (1H, s), 11.39 (1H, br s), 12.05 (1H, br s); MS (CI) 335 (M^+); Anal. calcd for $C_{14}H_{14}N_4O_6$: C, H; N, calcd 16.76; found 16.31.
- 4-{2-[Ethyl-(6-methyl-7-nitro-2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-ylmethyl)-amino]-acetylamino}-butyric acid methyl ester (**6b**). Red-orange solid, mp 153 - 157 °C (dec); ^{1}H NMR (400 MHz, DMSO) δ 1.63 (2H, m), 2.25 (2H, t), 2.63 (2H, t), 3.06 (6H, m), 3.51 (3H, s), 3.67 (2H, s), 7.69 (1H,s), 7.83 (1H, t), 11.47 (1H, s), 12.07 (1H, s); MS (ES) 420 (M^+); Anal. calcd for $C_{18}H_{21}N_5O_7$ 2 H_2O: C, N, H- calcd 5.53; found 4.96.
- [Ethyl-(6-methyl-7-nitro-2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-ylmethyl)-amino]-acetic acid (**7a**).). Tan solid. ^{1}H NMR (400 MHz, DMSO) δ 3.06 (2H, t), 3.16 (2H, s), 3.68 (2H, t), 4.05 (2H, br s), 7.86 (1H, s), 11.62 (1H, br s), 12.27 (1H, s); MS (CI) 321 (M^+); Anal calcd for $C_{11}H_{12}N_4O_6$ H_2O: C, H, N.
- 4-{2-[Ethyl-(6-methyl-7-nitro-2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-ylmethyl)-amino]-acetylamino}-butyric acid (**7b**). Brown solid, mp 263 - 265 °C (dec); ^{1}H NMR (400 MHz, DMSO) δ 1.60 (2H, m), 2.15 (2H, t), 2.62 (2H, br s), 3.07 (6H, m), 3.64 (2H, s), 7.66 (1H, s), 7.82 (1H, br s), 11.73 (1H, br s), 11.98 (1H, br s); MS (CI) 406 (M^+); Anal. calcd for $C_{17}H_{19}N_5O_7$ H_2O: C, H, N.
- 4-{2-[Ethyl-(6-methyl-7-nitro-2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-ylmethyl)-amino]-acetylamino}-N-[2-(4-hydroxy-phenyl)-ethyl]butyramide (**9**). ^{1}H NMR (400 MHz, DMSO) δ 1.58 (2H, br t), 1.99 (2H, br t), 2.60 (2H, br t), 2.67 (2H, br s), 3.0-3.2 (8 H, m), 3.66 (2H, br s), 6.60 (2H, d), 6.91 (2H, d), 7.68 (1H, s), 7.81 (sH, br s), 9.10 (1H, br s), 11.44 (1H, br s), 12.05 (1H, br s).
- 4-{2-[Ethyl-(6-methyl-7-nitro-2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-ylmethyl)-amino]-acetylamino}-N-[2-(4-hydroxy-3-iodo-^{125}I-phenyl)-ethyl]butyramide (**10**). Purified by HPLC (Alltech Econosil C-18; 0 - 5 min 10% CH_3CN in 0.05 M potassium formate buffer (pH 4); 5 - 95 min 10 - 40% gradient CH_3CN in buffer; 95 - 160 min 40% CH_3CN in buffer.) Elution of [^{125}I]-product at 68 min.

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